UNIVERSITY
OF TASMANIA

USING THE MUCOSAL RESPONSE TO RECOMBINANT *Neoparamoeba perurans* ATTACHMENT PROTEINS TO DESIGN AN EXPERIMENTAL VACCINE AGAINST AMOEBIC GILL DISEASE (AGD)

by

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Mucosal immune responses to *Neoparamoeba perurans*

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ABBREVIATIONS

2-D	two dimensional
AGD	amoebic gill disease
ANOVA	analysis of variance
ASC	antibody secreting cell
BCA	bicinchoninic acid
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
b.w.	body weight
CTL	cytotoxic T lymphocyte
d	day
df	degrees of freedom
DNA	deoxyribonucleic acid
DNP	dinitrophenol
ELISA	enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FITC	fluorescein isothiocyanate
G	gauge
g	gravity
g	gram
h	hour
HRP	horseradish peroxidase
HSWB	high salt wash buffer
ICC	immunocytochemistry
IgG	immunoglobulin G
IgM	immunoglobulin M
IgT	immunoglobulin T
i.p.	intraperitoneal
kDa	kilodalton
KLH	keyhole limpet haemocyanin
L	litre
L-15	L-15 Medium (Leibovitz) for cell culture
LB	Luria Bertani media
LC MS/MS	liquid chromatography tandem mass spectrometry
LSWB	low salt wash buffer
M	mol
mAb	monoclonal antibody
MBP	mannose-binding protein
mg	milligram
MHC	Major histocompatibility complex
min	minute
mL	millilitre
mm	millimetre
mM	micromole
mRNA	messenger ribonucleic acid

n	number of samples
nm	nanometer
NAPS	nucleic acid preservation solution
NCBI	National Centre for Biotechnology Information
NCMCRS	National Centre for Marine Conservation and Resource Sustainability
OD	optical density
OIE	World Organization for Animal Health
p.a.	peranal
PAMP	pathogen associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PRR	pattern recognition receptor
PSN	penicillin – streptomycin – neomycin
PVDF	polyvinylidene difluoride
s	second
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SE	standard error
r	recombinant
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
TBS	tris-buffered saline
TLR	Toll- like receptor
TMB	3,3´,5,5´-tetramethyl benzidine
V	Volts
W	Watts
WB	western blot
x	times
μm	micrometre
μL	microlitre

EXECUTIVE SUMMARY

Amoebic gill disease (AGD) is the main disease affecting the Tasmanian salmonid industry and the condition has also been described in other major salmon and trout producing countries. AGD is caused by *Neoparamoeba perurans*, and outbreaks of the disease appear during the marine grow-out phase, in particular when water temperature rises. Some characterisation of the host immune response against the parasite has been achieved through gene expression studies and through others investigations which focused on antibody responses against *N. perurans*, particularly IgM. A variety of treatments have been tested, but currently the only treatment option widely used in Tasmania is freshwater bathing, which represent a high economic burden for the industry. Therefore, the development of a vaccine remains a high priority for salmon producers and different types of vaccines have been previously tested against AGD without success.

In order to develop a potentially successful vaccine strategy, a better understanding of the antibody immune response associated with the disease is necessary. To address this general objective, the followings aims were studied in this thesis:

- Investigate the mucosal and systemic immune response of Atlantic salmon against *N. perurans*, the causative agent of AGD.
- Investigate mucosal and systemic anti-*N. perurans* antibody responses to a recombinant putative attachment protein of the amoeba, first identified by the generation of a cDNA library from the parasite.
- Investigate vaccine formulations for AGD, using the recombinant protein described above.
- Investigate other mucosal components potentially involved in the host response against *N. perurans*.

This thesis presents the results obtained from several different experiments aimed at addressing the above stated aims. Firstly, an experiment where the immune responses of Atlantic salmon were assessed at transcription and antibody production levels, after repeated infections with *N. perurans.* Secondly, an experiment where immune responses were assessed after a single infection and fish were fed commercially

developed diets containing immunostimulants. We showed that antibody levels do not always correlate with mRNA transcription levels identified in AGD gill lesions, which is possibly explained by weak correlations existing between protein and mRNA abundances in cells and tissues. Additionally, we demonstrated that the use of immunostimulants containing diets did not affect the levels of serum or skin mucus IgM and were unable to induce IgM and IgT transcription at the site of AGD infection.

Following from this experiment; the systemic and mucosal immune responses of Atlantic salmon were studied using two protein-hapten antigens. This study aimed at evaluating the best delivery method of antigens to be used in the testing of a vaccine candidate in subsequent experiments. The results showed that i.p. injection of immunogens emulsified in FCA was the best delivery method for inducing systemic and mucosal antibody responses.

We described the production of a recombinant protein named *r*22C03, identified as a mannose-binding protein-like (MBP-like) similar to attachment factors of other amoebae, and a putative attachment factor of *N. perurans.* This protein was capable of inducing systemic and mucosal antibody responses against the amoebae and both systemic and mucosal antibodies produced were able to bind the surface of formalin-fixed *N. perurans*. The recombinant protein was then tested as a vaccine candidate against AGD, following the rationale that by using functional antibodies present in mucosal surfaces, the putative attachment factor of *N. perurans* might be blocked and the severity of AGD could potentially be reduced. Fish were immunised with *r*22C03 using two different vaccination strategies and then challenged with the parasite. A strong antibody response against the recombinant protein was observed in serum and mucosal surfaces of vaccinated salmon, but no differences in survival curves or size of lesion in the gills were observed. However, a concurrent infection with *Yersinia ruckeri* was present during the experiment, and even though the simultaneous presentation of both pathogens could represent a situation more closely related to infection patterns observed on commercial farms, survival results obtained after the parasite challenge had to be examined with caution in the context of vaccine efficacy against *N. perurans*.

Following from the unsuccessful challenge, nanoLC-MS/MS and proteomics analyses were used on skin and gill mucus of AGD-affected fish, as a tool to identify the changes in the proteome of mucus after repeated infection with amoebae. Proteins that have been previously related to gene expression in AGD-affected gills as well as proteins that have not been previously described in AGD-affected fish were identified and it was proposed that future research should focus on better understanding the role these components play in the response against infection with *N. perurans.*

This thesis provided further understanding into the mucosal responses to AGD. However, the role mucosal antibodies play in responses against AGD cannot be completely comprehended until the study of IgT responses in AGD-affected fish can be completed, as it has been hampered by the lack of available reagents. Finally, adjuvants that have been designed specifically to elicit mucosal responses need to be fully tested in AGD vaccine formulations.